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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

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Plants having modified growth characteristics and a method for making the same

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Plants having modified growth characteristics and a method for making the same

The present invention concerns a method for modifying plant growth characteristics. More specifically, the present invention concerns a method for modifying plant growth characteristics by modulating expression of a nucleic acid sequence encoding a zinc-finger protein and/or activity of a zinc-finger protein in a plant. The present invention also concerns plants having modulated expression of a nucleic acid sequence encoding a zinc-finger protein and/or modulated activity of a zinc-finger protein, which plants have modified growth characteristics relative to corresponding wild type plants.

Given the ever-increasing world population, it remains a major goal of agricultural research to improve the efficiency of agriculture and to increase the diversity of plants in horticulture. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant. Such technology has led to the development of plants having various improved economic, agronomic or horticultural traits. A trait of particular economic interest is high yield.

Since the discovery of zinc finger domains in the *Xenopus* transcription factor TFIIIA, hundreds of proteins have been described encoding this nucleic acid binding domain in a variety of organisms. In the genome of *Arabidopsis thaliana* more than 200 zinc finger proteins have been predicted. The canonical ZF (zinc finger) sequence C2H2 (CX2_4CX3FX5LX2HX3-5H) contains 2 cysteines and 2 histidines that coordinate a zinc atom, creating a compact nucleic acid-binding domain. It has also been suggested that a Zn-centred domain could be used in a protein interaction, e.g. in protein kinase C. Multiple zinc finger domains can be found as tandem arrays in ZF proteins. In addition other domains can also be present. Thes 2 characteristics, number of ZF and presence of other domains are used as criteria to classify this proteins. In addition to the conserved zinc ligand residues a number of other positions are also important for the structural integrity of the C2H2 zinc fingers. The best conserved position is found four residues after the second cysteine; it is generally an aromatic or aliphatic residue. Plant zinc-finger proteins are characterized by long spacers of diverse lengths between adjacent fingers and a highly conserved sequence, QALGGH, located within a putative DNA-contacting surface of each finger

Zinc fingers domains are present in many type of proteins and binding to both DNA and RNA binding has been shown for some members of this family. However the majority are DNA binding transcription factors. They can act as transcriptional activators such as SP1 (Kadonaga *et al.*, Cell 51 (6) 1079-10902 (1987)) and ADR1 (Blumberg *et al.*, Nature 328 (6129) 443-445

(1987)) or transcriptional repressors such as Kruppel and ZF5 or the oncogene Wilm's tumor factor 1 '(WT1). Many of them have been shown to play diverse roles in development some of them affecting organ specification, or cell proliferation.

In plants only very zing finger proteins have been analysed. Mutational analysis, expression studies, forward genetics or complementation assays have been used to defined the function of these genes. The few ZF genes to which a function has been assigned support a role in plant developmental processes linked to organ specifications, particularly of flowers, leaves and lateral roots (Colasanti *et al.*, Cell 93 (4) 593-603 (1998); Luo *et al.*, PNAS 96 (1) 296-301 (1999); Takatsuji, Plant Mol Biol. 39 (6) 1073-1078 (1999)). The best-characterized *Arabidopsis* ZF protein is Superman, which encodes a protein encoding a single C2H2 domain, involved in floral organogenesis. Overexpression of the arabidopsis sup gene in arabidopsis plants suppresses development of stamens and petals (Yun *et al.*, Plant Cell Physiol 43 (1) 52-57 (2002). When the *Arabidopsis* gene is overexpressed in rice the flowers showed reduced stamen number and other abnormalities in flower organs. Rice plants with high expression of the *Arabidopsis* superman gene showed juvenile death and dwarf plants.

Another example shows that ectopic expression of zinc-finger proteins leads to disruption of development, thus overexpression of atZAT10 in tobacco resulted in plants dwarfism, abnormal leaf phenotypes, and early flowering (Dinkins *et al.*, Plant Cell Physiol.43 (7) 743-750 2002).

Other plant proteins with one single zinc finger C2H2 have been shown to play a role in development of other organs, such as the seeds, via the mechanism of Fertilization Independent seed development (Fis) (Luo *et al.*, PNAS 96 (1) 296-301 (1999)), in the leaves by SERRATE Prigge and Wagner *et al.*, Plant Cell 13 (6) 1263-1279 (2001), in the shoot, At ZFP1 (Chrispeels *et al.*, Plant Mol Biol. 42 (2) 279-290 (2000)). Misexpression of members of the AtZF1, characterized by a single C2H2 domain and a short C-term leucine domain has profound consequences for plant morphology (Tague *et al.*, Plant Mol Biol. 32 785-796 (1997)). Members of other type of zinc finger proteins, encoding a yabby domain, defined by a combination of a zinc finger and a helix-loop-helix domain, have been implicated in floral organ development. *Arabidopsis* proteins with yet another type of zinc finger domain, a Dof/MOA, of type of those found in steroid hormone receptors, have been propose to act on a maternal switch that controls seed germination. Several anther specific zinc finger proteins in petunia are presumed to be involved in the regulation of gametogenesis.

A subset of plant zinc finger proteins characterized by having 2 zinc finger domains have been primarily implicated in the response of plants to various stresses (Sakamoto *et al.*, Gene 248 (1-2) 23-32 (2000)). Expression of AZF1, AZF2, AZF3 and STZ/ZAT10 is upregulated by water

stress or high salt exposure. All of them except AZF2 are also upregulated by cold stress. The soybean SCOF-1 gene is also upregulated by ABA and cold but not dehydration or salinity.
All this ZF proteins play a role in the regulation of gene expression under abiotic stresses however their mechanism of action can be very different. STZ/ZAT10 has been propose to be transcriptional repressor and has been shown to act as such on the promoter of a stress response gene, rd29A. This repression is thought to be mediated through direct DNA binding (Lee *et al.*, EMBO J 21 (11) 2692-2702 (2002)). Very different is the regulation of stress genes by the soybean homologue SCOF-1, which is regarded as a transcriptional activator. Activation is thought to be through a protein-protein interaction rather than direct DNA binding to the promoter sequences. SCOF-1 binds to the transcription factor that makes the direct contact with the promoter sequences, leading to enhancement of gene expression of stress genes (Kim *et al.*, Plant J. 25 (3) 247-259 (2001)). Despite their different mechanism of actions, both STZ and SCOF-1 have been used to enhanced abiotic stress tolerance when overexpress in an organsim, STZ increases salt tolerance in yeast (Lipunner *et al.*, J Biol Chem. 271 (22) 12859-12866 (1996)) and the SCOF-1 gene under control of the CaMV 35 S promoter, enhances cold tolerance in Arabidopsis (Kim *et al.*, Plant J. 25 (3) 247-259 (2001)).

Regulation of ZF proteins in plants has not been studied in detail. Clearly different members of the ZF protein family are differently regulated. At the transcriptional level mRNA levels can be regulated differentially according to tissue, cell type or in response to biotic and abiotic stresses and also developmental regulation has been shown for some members. Another level of regulation proposed has been at the posttranscriptional level. A further level of regulation of the activity is by localization of the protein. The site of action for many of these proteins is the nuclei, some of them have a nuclear localization signal to direct the protein to the nuclei (Kim et al. 2001). A further level of regulation of ZF proteins could be the modification of the relation between the site of ZF protein versus the site of action. Another zinc finger protein that controls transition to flowering in maize, id1, has been shown to act in a non-cell-autonomous fashion, i.e. is works on another cell than the cell in which it is synthesised or present (Colasanti *et al.*, Cell 93 (4) 593-603 (1998)). These mechanisms can all be used to alter the activity of a Zinc-finger protein in a plant cell. A further level of regulation maybe the control of the stoichiometry and identity of the subunits present in the protein complexes in which they exert their activity. Zinc finger proteins have been described to interact with various proteins, eg with other transcription factor which can belong to the zinc finger or to other type of family.
Still another level of regulation of zinc-finger proteins is via the upstream elements of the signalling cascade, which influences the activity of zinc-finger transcription factors. For the STZ transcription factor two such elements have already been identified, namely SOS-2 and LOS-2 (Zhu et al, Arizona university).

Taken all together all the plant phenotypes described to date and obtained by modulating the expression of a gene encoding a zinc finger protein and/or modulating the activity of zinc finger proteins in the plant, whether the gene has been overexpressed or mutated, showed abnormal growth characteristic, not desired for a crop, except for the cold tolerance enhancement shown by the transgenic plants expressing SCOF-1. Therefore, it has not yet been demonstrated how to use a zinc-finger protein or a gene encoding a zinc-finger protein, such zinc-finger protein being whether a single zinc-finger domain protein or a double zinc-finger domain protein, to improve the growth characteristics (other than cold-tolerance in case the zinc-finger protein is a double zinc finger protein) or yield of a plant, to make it economically more valuable.

In the present invention it is described how plant growth characteristics may be modified by modulating expression in a plant of a nucleic acid encoding a zinc finger protein.
It has now been found that not only in stress conditions, for example in cold stress conditions, but also in normal or even optimal growing conditions, plant growth characteristics may be modified by modulating expression in a plant of a nucleic acid encoding a zinc finger protein, resulting in plants having improved economic value. It has now been found that also plant growth characteristics, other than cold stress tolerance, may be modified by modulating expression in a plant of a nucleic acid encoding a zinc-finger protein, such as increased biomass, increased plant height, increased number of panicles, increase number of filled seeds, increased total seed yield per plant and increased harvest index and yield stability. It has now been found that plant growth characteristics may be modified by modulating expression in a plant of a nucleic acid encoding a zinc-finger protein, wherein the plant is a monocot, such as a cereal, for example in rice.

Therefore according to a first embodiment of the present invention there is provided a method for modifying the growth characteristics of a plant, comprising modulating expression in a plant of a nucleic acid sequence encoding a zinc-finger protein and/or modulating activity in a plant of a zinc finger protein. In a particular embodiment of the invention, the growth characteristic is other than cold-tolerance. In a further embodiment of the invention, the growth characteristic is improved. In another embodiment of the invention the plant with modified and/or improved growth characteristics is a monocot, such as a cereal.

Modulating (enhancing or decreasing) expression of a nucleic acid sequence encoding a zinc-finger protein or modulation of the activity of the zinc-finger protein itself encompasses altered expression of a gene and/or altered levels of a gene product, namely a polypeptide, in specific cells or tissues.

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Advantageously, modulation of expression of a nucleic acid sequence encoding a zinc-finger protein and/or modulation of activity of the zinc-finger protein itself may be effected by chemical means, i.e. by exogenous application of one or more compounds or elements capable of modulating activity of the zinc-finger protein and/or capable of modulating expression of a zinc-finger gene (which may be either an endogenous gene or a transgene introduced into a plant). The exogenous application may comprise contacting or administering cells, tissues, organs or organisms with the gene product or a homologue, derivative or active fragment thereof and/or to antibodies to the gene product. Such antibodies may comprise "plantibodies", single chain antibodies, IgG antibodies and heavy chain camel antibodies, as well as fragments thereof. Modulation of expression of a nucleic acid sequence encoding a zinc-finger protein and/or modulation of activity of the zinc-finger protein itself may also be effected as a result of decreased levels of factors that directly or indirectly activate or inactivate a zinc-finger protein. Additionally or alternatively, contacting or administering cells, tissues, organs or organisms with an interacting protein or to an inhibitor or activator of the gene product provides another exogenous means for modulation of expression of a nucleic acid sequence encoding a zinc-finger protein and/or for modulation of activity of the zinc-finger protein itself.

Therefore, according to one aspect of the present invention, there is provided a method for modifying the growth characteristics of a plant, comprising exogenous application of one or more compounds or elements capable of modulating expression of a zinc-finger gene and/or capable of modulating activity of a zinc-finger protein.

Additionally or alternatively, and according to a preferred embodiment of the present invention, modulation of expression of a nucleic acid sequence encoding a zinc-finger protein and/or modulation of activity of the zinc-finger protein itself may be effected by recombinant means. Such recombinant means may comprise a direct and/or indirect approach for modulation of expression of a nucleic acid sequence and/or activity of a protein.

For example, an indirect approach may comprise introducing, into a plant, a first nucleic acid sequence capable of modulating activity of the protein in question (a zinc-finger protein) and/or capable of modulating the expression of the gene in question (a gene encoding a zinc-finger protein). The zinc-finger gene or protein may be wild type, i.e. the native or endogenous nucleic acid or polypeptide. Alternatively, it may be a protein or nucleic acid derived from the same or another species. The nucleic acid/gene may then be introduced into a plant as a transgene, for example by transformation. This transgene may be substantially modified from

its native form in composition and/or genomic environment through deliberate human manipulation. Also encompassed by an indirect approach for modulating activity of a zinc-finger protein and/or expression of a zinc-finger gene is the inhibition or stimulation of regulatory sequences that drive expression of a native gene or transgene. Such regulatory sequences may be introduced into a plant.

A direct and preferred approach on the other hand comprises introducing into a plant a nucleic acid sequence encoding a zinc-finger protein or a homologue derivative or active fragment thereof. The nucleic acid sequence may be introduced into a plant by, for example, transformation. The nucleic acid sequence may be derived (either directly or indirectly (if subsequently modified)) from any source provided that the sequence, when expressed in a plant, leads to modulated expression of a zinc-finger protein nucleic acid/gene or modulated activity of a zinc-finger protein. The nucleic acid sequence may be isolated from a microbial source, such as, yeast or fungi, or from a plant, algal or animal (including human) source. This nucleic acid may be substantially modified from its native form in composition and/or genomic environment through deliberate human manipulation. The nucleic acid sequence is preferably a nucleic acid sequence obtained from a plant, whether from the same plant species as to the plant where it is introduced or from a different plant species. In a further preferred embodiment, the gene is originating from a dicot and the transformed plant is a monocot, for example an *Arabidopsis* gene into a cereal. Further preferably, the nucleic acid sequence is isolated from *Arabidopsis thaliana*. More preferably, the nucleic acid is essentially similar to a nucleic acid as represented by SEQ ID NO 1 or a portion of SEQ ID NO 1, or is a nucleic acid sequence encoding an amino acid sequence essentially similar to an amino acid sequence as represented by SEQ ID NO 2 or SEQ ID NO 4 or a homologue, derivative or active fragment thereof.

Therefore, according to one aspect of the present invention, there is provided a method for the production of plants, having modified growth characteristics, comprising introducing, into a plant, a nucleic acid sequence capable of modulating activity of a zinc finger protein and/or capable of modulating expression of a snip-like gene.

The term zinc-finger nucleic acid sequence/gene, as defined herein, refers to a nucleic acid sequence essentially similar to a nucleic acid sequence/gene as represented by SEQ ID NO 1 or a portion thereof, or to nucleic acid sequences capable of hybridising with either SEQ ID NO 1, and to nucleic acid sequences encoding an amino acid sequence/protein essentially similar to an amino acid sequence/protein represented by SEQ ID NO 2 or to homologues, derivatives or active fragments thereof.

Advantageously, the method according to the present invention may also be practised using portions of a sequence represented by SEQ ID NO 1 or by using sequences that hybridise (preferably under stringent conditions) to SEQ ID NO 1 or by sequences encoding homologues, derivatives or active fragments of a sequence according to SEQ ID NO 2.

The term zinc-finger amino acid sequence/protein encompasses proteins having at least one zinc-finger domain. Preferably the zinc-finger proteins to use in the methods of the present invention have at least two zinc-finger domains. Further preferably the zinc-fingers protein to use in the methods of the present invention have two zinc-finger domains and an nuclear localization signal. Preferably the zinc finger protein used in the methods of the present invention belongs to the same gene family as the salt tolerant zinc finger protein (STZ) of Arabidopsis thaliana, or the homologues thereof in other plant species. Also the name ZAT10 was used to identify the STZ zinc-finger protein of *Arabidopsis thaliana*. Further preferably, the term zinc-finger amino acid sequence/protein refers to a protein encoding an amino acid sequence/protein essentially similar to an amino acid sequence/protein represented by SEQ ID NO 2 or to homologues, derivatives or active fragments thereof.

Advantageously, the method according to the present invention may also be practised using the protein represented by SEQ ID NO 2 or by using homologues, derivatives or active fragments of a sequence according to SEQ ID NO 2.

A zinc-finger protein encompasses a zinc-finger protein essential similar to a protein as presented by SEQ ID NO 2. A protein essential similar to a protein as presented by SEQ ID NO 2 encompasses a protein as presented in SEQ ID NO 2 itself or, homologues, derivatives and functional fragments of SEQ ID NO 2. A zinc-finger gene encompasses a gene essential similar to a gene as presented by SEQ ID NO 1. A gene essential similar to a gene as presented by SEQ ID NO 1 encompasses a gene as presented in SEQ ID NO 1 itself or, homologues, derivatives and functional fragments of SEQ ID NO 1. The term "essentially similar to" also includes at least a part or a portion of sequences SEQ ID NO 1 or SEQ ID NO 2; a complement of the sequences 1 or 2; RNA, DNA, a cDNA or a genomic DNA corresponding to the sequences 1 or 2; a variant of the gene or protein due to the degeneracy of the genetic code; a family member of the gene or protein; an allelic variant of the gene or protein; and different splice variant of the gene or protein and variants that are interrupted by one or more intervening sequences. Advantageously, nucleic acids or proteins essentially similar to the proteins and nucleic acids according to the invention may be used in the methods of the present invention.

According to a preferred feature of the present invention, the nucleic acid sequence capable of modulating expression of a zinc-finger gene or modulating activity of a zinc-finger protein is a nucleic acid sequence homologous to SEQ ID NO 1. This homologue is preferably a plant homologue, i.e. a nucleic acid sequence obtained from a plant, whether from the same plant species or different. Further preferably, the nucleic acid is a nucleic acid sequence encoding a zinc-finger protein, derivative or active fragment thereof. More preferably, the nucleic acid sequence is isolated from a dicot, more preferably from *Arabidopsis thaliana*. Further preferably the protein is a STZ protein or a homologue, derivative or functional fragment thereof. Most preferably, the nucleic acid sequence capable of modulating expression of a gene essentially similar to SEQ ID NO 1 or activity of a protein essentially similar to SEQ ID NO 2 is a nucleic acid sequence as represented by SEQ ID NO 1 or a homologue, derivative or active fragment thereof or a nucleic acid sequence encoding an amino acid sequence represented by SEQ ID NO 2 or a homologue, derivative or active fragment thereof. Preferably, the nucleic acid sequence represented by SEQ ID NO: 1 is overexpressed in a plant. However, it should be clear that the applicability of the invention is not limited to use of the nucleic acid represented by SEQ ID NO 1 nor to the nucleic acid sequence encoding the amino acid sequence of SEQ ID NO 2, but that other nucleic acid sequences encoding homologues, derivatives or active fragments of SEQ ID NO: 1 or SEQ ID NO: 2 may be useful in the methods of the present invention.

Therefore according to another aspect of the present invention, there is provided a method for modifying the growth characteristics of plants, comprising introducing into a plant a homologue, derivative or active fragment of a nucleic acid sequence capable of modulating zinc-finger gene expression and/or protein activity in a plant, preferably a homologue, derivative or active fragment of a sequence represented by SEQ ID NO 2, such as a homologue, derivative or active fragment as described below.

Methods for the search and identification of zinc-finger protein homologues, more particularly STZ zinc-finger homologues would be well within the realm of a person skilled in the art. Methods for the alignment of sequences for comparison are well known in the art, such methods include GAP, BESTFIT, BLAST, FASTA and TFASTA. GAP uses the algorithm of Needleman and Wunsch (J. Mol. Biol: 48: 443-453, 1970) to find the alignment of two complete sequences that maximises the number of matches and minimises the number of gaps. The BLAST algorithm calculates percent sequence identity and performs a statistical analysis of the similarity between the two sequences. The software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information.

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The analysis of a gene family can be carried out by sequence similarity analysis. To perform this analysis one can use standard programs for multiple alignments e.g. Clustal W. This analysis can be done on the full length sequence or based on a comparison of certain regions such as conserved domains.

Over 200 family members of zinc-finger proteins were discovered in the *Arabisopsis* genome. The STZ gene and protein as represented in SEQ ID NO 1 and 2 were previously published in the database under the MIPS accession number At1g27730 and several other cDNA's, isolated from other tissues or different developmental stages of Arabisopsis have been reported and encode the same protein as presented in SEQ ID NO 2 (e.g. the sequences with the Genbank accession number AY034998, NM_102538, AC12375, X95573 (AtSTZ), AY063006, X98671 (AtZAT10), X98670, AF250336). These isolates illustrate the differential expression and of the STZ gene in different plant tissues at different developmental stages.The differential regulation of these different cDNA's is reflected by the differences at the 5'UTR and the 3'UTR regions of these cDNA's.

The MIPS database contains the sequence of the *Arabidopsis thaliana* genome with prediction and functional annotation of the proteins encoded. Searching this database with the STZ gene as presented in SEQ ID NO 1 (MIPS accession number At1g27730), showed that in the *Arabidopsis* genome there are 2 very close homologues to SEQ ID NO 2, At5g43170 and at5g04340, and 3 other with very high similarity (X**% of similarity) At3g19580, At5g67450, At3g49930. The closest homologues genes are spread in over 3 chromosomes 1, 3 and 5. Additionally, another gene At5g43160 is found contiguous in the chromosome to the closest homologous protein At5g43170. The sequence similarity between At5g43160 and At5g43170 suggests that they are related in function. The presence of both genes at the same chromosomic location further strengthens this theory. It is well established that chromatin structures influences gene expression. Additionally, it has been described that genes related to the same function or physiological process can be arrange in the same chromosomic location to constitute higher levels of gene regulation like for example the operons found in bacteria. Though such operon-like organizations have not been clearly established in plants examples exist were genes related to the same physiological process are found at the same chromosomic locations, like the CBF1, CBF2, and CBF3. In this example these genes are also related in sequence.

Homologues of the STZ protein of Arabidopsis to use in the present invention are also identified in other plant species. Protein homologous to the STZ protein as presented in SEQ ID NO 2 were found in a variety of dicots plants, Arabidopsis, Medicago, petunia, soybean, tobacco, brassica rapa, and also in monocots like rice, wheat or sugarcane. Examples of such

homologues, that are the closest homologues of the STZ protein from another organism as available at present in the public databases, are the sequences as published in Genbank under the accession number: AAF24959, NP_174094.1, CAA67229.1, BAC43454.1, NP_196054.1, AAM67193.1, NP_199131.1, AAD26942.1, AF119050_1 ,CAB77055.1, BAA05079.1 (Petunia), T09602 SCOF-1, T01985 , BAA05077.1, BAA05076.1, NP_188692.1, NP_201546.1, T14408 , T14409 ZFP1 (rice), BAA05078.1, NP_190562.1, S39045 WZF1 (wheat) , AAK01713.1 AF332876_1 , NP_182037.1, BAC43008.1,

It is expected that as more sequences of the genomes of other plants will become available, still many other homologues of the STZ protein shall be identifiable by sequences alignment with SEQ ID NO 1 or SEQ ID NO 2.

In a particular embodiment of the present invention, the protein encoded by zinc-finger gene and/or zinc-finger protein to be used in the methods of the present invention shows a sequence homology that is no smaller than the sequence identity of SEQ ID NO 2 with Os_BAB67879 (or with OS_AF332876, which is 36% identity) and falls within the phylogenetic tree as represented in Figure 4A. In another embodiment of the present invention the protein encoded by a zinc-finger gene and/or the zinc-finger protein to be used in the methods of the present invention, shows a sequence homology that is larger than the sequence identity of SEQ ID NO 2 with Gm_T09602 and falls within the boundaries of ARA_At1g27730 and Gm_T09602 of the phylogenetic tree as represented in Figure 4B. A similar tree structure, to express the sequence similarity and identity of (newly found) homologues of the STZ protein of the present invention, can be made using the following program and parameters. The alignment and phylogenetic tree of figure 4 was made using clustal W present in the VNTI (version 5.0) program with Gap opening penalty 10 and Gap extention 5.

"Homologues" of a zinc-finger protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes having amino acid substitutions, deletions and/or insertions relative to the unmodified protein in question and having similar biological and functional activity as the unmodified protein from which they are derived. To produce such homologues, amino acids of the protein may be replaced by other amino acids having similar properties (such as similar hydrophobicity, hydrophilicity, antigenicity, propensity to form or break α-helical structures or β-sheet structures). Conservative substitution tables are well known in the art (see for example Creighton (1984) Proteins. W.H. Freeman and Company). The homologues useful in the method according to the invention have at least 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52, 54, 56, 58, % sequence identity or similarity (functional identity) to the unmodified protein, alternatively at least 60, 62, 64, 66, 68,% sequence identity or similarity to an unmodified protein, or alternatively at least 70, 72, 74, 76, 78% sequence identity or

similarity to an unmodified protein. Typically, the homologues have at least 80, 82, 84% sequence identity or similarity to an unmodified protein, preferably at least 85, 86, 88% sequence identity or similarity, further preferably at least 90, 92, 94, 96, 98% sequence identity or similarity to an unmodified protein, most preferably at least 95% sequence identity or similarity to an unmodified protein.

Preferred homologues to use in the methods of the present invention are homologues showing 35% or more sequence identity with the STZ zinc-finger protein as presented in SEQ ID NO 2. Preferred homologues of the STZ zinc-finger protein as presented in SEQ ID NO 2 from other plant species, are the closest homologues found in the genome of that plant species and any other gene from that plant species which shows 35% or more homology to that closest homologue. Advantageously, the members of the same gene family as SEQ ID NO 1 from the same (Arabidopsis) or from different plant species can be used in the methods of the present invention.

Two special forms of homology, orthologous and paralogous, are evolutionary concepts used to describe ancestral relationships of genes. The term "paralogous" relates to gene-duplications within the genome of a species leading to paralogous genes. The term "orthologous" relates to homologous genes in different organisms due to ancestral relationship. The term "homologues" as used herein also encompasses paralogues and orthologues of the proteins useful in the methods according to the invention.

In a particular embodiment of the present invention proteins of one plant species (for example Arabidopsis) are introduced in another plant species (for example rice) such that there is enough difference in a way that normal activity the protein and/or protein complexes in which zinc-finger proteins work are disrupted. Therefore their could be an advantage in the use of proteins, genes encoding such proteins on the one hand and host plants from different plant origin on the other hand. For example, it has been shown in the present invention that plant growth characteristics can be improved by intriduction of a zinc-finger gene or protein from a dicot into a monocot.

"Substitutional variants" of a protein are those in which at least one residue in an amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues, and deletions will range from about 1-20 residues. Preferably, amino acid substitutions comprise conservative amino acid substitutions.

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"Insertional variants" of a protein are those in which one or more amino acid residues are introduced into a predetermined site in said protein. Insertions can comprise amino-terminal and/or carboxy-terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino- or carboxy-terminal fusions, of the order of about 1 to 10 residues. Examples of amino- or carboxy-terminal fusion proteins or peptides include the binding domain or activation domain of a transcriptional activator as used in the yeast two-hybrid system, phage coat proteins, $(histidine)_6$-tag, glutathione S-transferase-tag, protein A, maltose-binding protein, dihydrofolate reductase, Tag•100 epitope, c-myc epitope, FLAG®-epitope, lacZ, CMP (calmodulin-binding peptide), HA epitope, protein C epitope and VSV epitope.

"Deletion variants" of a protein are characterised by the removal of one or more amino acids from the protein. Amino acid variants of a protein may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. Methods for the manipulation of DNA sequences to produce substitution, insertion or deletion variants of a protein are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA are well known to those skilled in the art and include M13 mutagenesis, T7-Gen *in vitro* mutagenesis (USB, Cleveland, OH), QuickChange Site Directed mutagenesis (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

The term "derivatives" refers to peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise substitutions, deletions or additions of naturally and non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the protein as presented in SEQ ID NO 2. "Derivatives" of a zinc-finger protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise naturally occurring altered, glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the polypeptide. A derivative may also comprise one or more non-amino acid substituents compared to the amino acid sequence from which it is derived, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound to facilitate its detection, and non-naturally occurring amino acid residues relative to the amino acid sequence of a naturally-occurring protein.

"Active fragments" of a zinc-finger protein encompasses at least five contiguous amino acid residues of a protein, which residues retain similar biological and/or functional activity to the naturally occurring protein.

Advantageously, the method according to the present invention may also be practised using portions of a DNA or nucleic acid sequence. Portions of a DNA sequence refer to a piece of DNA derived or prepared from an original (larger) DNA molecule, which DNA portion, when expressed in a plant, gives rise to plants having modified growth characteristics. The portion may comprise many genes, with or without additional control elements, or may contain just spacer sequences etc.

The present invention also encompasses nucleic acid sequences capable of hybridising with a nucleic acid sequence encoding a zinc-finger protein, which nucleic acid sequences may also be useful in practising the methods according to the invention. The term "hybridisation" as defined herein is a process wherein substantially homologous complementary nucleotide sequences anneal to each other. The hybridisation process can occur entirely in solution, i.e. both complementary nucleic acids are in solution. Tools in molecular biology relying on such a process include the polymerase chain reaction (PCR; and all methods based thereon), subtractive hybridisation, random primer extension, nuclease S1 mapping, primer extension, reverse transcription, cDNA synthesis, differential display of RNAs, and DNA sequence determination. The hybridisation process can also occur with one of the complementary nucleic acids immobilised to a matrix such as magnetic beads, Sepharose beads or any other resin. Tools in molecular biology relying on such a process include the isolation of poly (A+) mRNA. The hybridisation process can furthermore occur with one of the complementary nucleic acids immobilised to a solid support such as a nitro-cellulose or nylon membrane or immobilised by e.g. photolithography to, for example, a siliceous glass support (the latter known as nucleic acid arrays or microarrays or as nucleic acid chips). Tools in molecular biology relying on such a process include RNA and DNA gel blot analysis, colony hybridisation, plaque hybridisation, *in situ* hybridisation and microarray hybridisation. In order to allow hybridisation to occur, the nucleic acid molecules are generally thermally or chemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridisation is influenced by conditions such as temperature, salt concentration and hybridisation buffer composition. High stringency conditions for hybridisation include high temperature and/or low salt concentration (salts include NaCl and Na_3-citrate) and/or the inclusion of formamide in the hybridisation buffer and/or lowering the concentration of compounds such as SDS (detergent) in the hybridisation buffer and/or exclusion of compounds such as dextran sulphate or

polyethylene glycol (promoting molecular crowding) from the hybridisation buffer. Conventional hybridisation conditions are described in, for example, Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York, but the skilled craftsman will appreciate that numerous different hybridisation conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid sequence. Specifically hybridising refers to hybridising under stringent conditions, i.e. at a temperature of 60°C followed by washes in 2XSSC, 0.1XSDS, and 1X SSC, 0.1X SDS. Sufficiently low stringency hybridisation conditions are particularly preferred for the isolation of nucleic acids heterologous to the DNA sequences of the invention defined supra. Elements contributing to heterology include allelism, degeneration of the genetic code and differences in preferred codon usage.

The methods according to the present invention may also be practised using an alternative splice variant of a nucleic acid sequence encoding a zinc-finger protein. The term "alternative splice variant" as used herein encompasses variants of a nucleic acid sequence in which selected introns and/or exons have been excised, replaced or added. Such variants will be ones in which the biological activity of the protein remains unaffected, which can be achieved by selectively retaining functional segments of the protein. Such splice variants may be found in nature or can be manmade. Methods for making such splice variants are well known in the art. Therefore according to another aspect of the present invention, there is provided, a method for modifying the growth characteristics of plants, comprising modulating expression in a plant of an alternative splice variant of a nucleic acid sequence encoding a zinc-finger protein and/or by modulating activity of a zinc-finger protein encoded by the alternative splice variant. Preferably, the splice variant is a splice variant of the sequence represented by SEQ ID NO 1.

Advantageously, the methods according to the present invention may also be practised using allelic variants of a nucleic acid sequence encoding a zinc-finger protein, preferably an allelic variant of a sequence represented by SEQ ID NO 1. Allelic variants exist in nature and encompassed within the methods of the present invention is the use of these natural alleles. The use of these allelic variants in particular conventional breeding programmes, such as in marker-assisted breeding is also encompassed by the present invention; this may be in addition to their use in the methods according to the present invention. Such breeding programmes sometimes require the introduction of allelic variations in the plants by mutagenic treatment of a plant. One suitable mutagenic method is EMS mutagenesis. Identification of allelic variants then takes place by, for example, PCR. This is followed by a selection step for selection of superior allelic variants of the sequence in question and which give rise to altered growth characteristics in a plant. Selection is typically carried out by monitoring growth

performance of plants containing different allelic variants of the sequence in question, for example, SEQ ID NO 1. Monitoring growth performance can be done in a greenhouse or in the field. Further optional steps include crossing plants in which the superior allelic variant was identified with another plant. This could be used, for example, to make a combination of interesting phenotypic features. Allelic variants also encompass Single Nucleotide Polymorphisms (SNPs), as well as Small Insertion/Deletion Polymorphisms (INDELs). The size of INDELs is usually less than 100 bp). SNPs and INDELs form the largest set of sequence variants in naturally occurring polymorphic strains of most organisms.

According to another aspect of the present invention, advantage may be taken of the nucleotide sequence capable of modulating expression of a nucleic acid encoding a zinc-finger protein in breeding programmes. For example, in such a programme, a DNA marker is identified which may be genetically linked to a gene capable of modulating expression of a nucleic acid encoding a zinc-finger protein in a plant, which gene may be a gene encoding the zinc-finger protein itself or any other gene which may directly or indirectly influence expression of the gene encoding a zinc-finger protein and/or activity of the zinc-finger protein itself. This DNA marker may then be used in breeding programs to select plants having altered growth characteristics.

The methods according to the present invention may also be practised by introducing into a plant at least a part of a (natural or artificial) chromosome (such as a Bacterial Artificial Chromosome (BAC)), which chromosome contains at least a gene encoding a zinc-finger protein, optionally together with one or more related gene family members. Therefore, according to a further aspect of the present invention, there is provided a method for modifying the growth characteristics of plants by expressing in a plant at least a part of a chromosome comprising at least a gene encoding a zinc-finger protein.

According to a preferred feature of the present invention, enhanced or increased expression of a nucleic acid encoding a zinc-finger protein is envisaged. Methods for obtaining enhanced or increased expression of genes or gene products are well documented in the art and include, for example, overexpression driven by a strong promoter, the use of transcription enhancers or translation enhancers.

Modulating gene expression (whether by a direct or indirect approach) encompasses altered transcript levels of a gene. Altered transcript levels can be sufficient to induce certain phenotypic effects, for example via the mechanism of cosuppression. Here the overall effect of overexpression of a transgene is that there is less activity in the cell of the protein encoded by

a native gene having homology to the introduced transgene. Therefore, according to another embodiment of the present invention, there if provided a method to alter growth characteristics in a plant, comprising decreasing expression of a gene encoding a zinc-finger protein or decreasing activity of a zinc-finger protein. Preferably that zinc-finger protein belongs the same family (or the same family in another plant species as the STZ protein from Arabidopsis thaliana. Preferably, this method comprises introduction of a zinc-finger encoding gene under the control of a constitutive promoter into a plant. Other examples of decreasing activity of a protein in a cell are well documented in the art and include, for example, downregulation of expression by anti-sense techniques, RNAi techniques, the use of ribozymes etc. Advantageously, the methods according to the present invention may also be practised by downregulation of a nucleic acid sequence encoding a zinc-finger protein. Plants having modified growth characteristics may be obtained by expressing a nucleic acid sequence encoding a zinc-finger protein in either sense or antisense orientation. Techniques for downregulation are well known in the art. The terms "gene silencing" or "downregulation" of expression, as used herein, refer to lowering levels of gene expression and/or levels of active gene product and/or levels of gene product activity. Such decreases in expression may be accomplished by, for example, the addition of coding sequences or parts thereof in a sense orientation (if it is desired to achieve co-suppression). Therefore, according to one aspect of the present invention, the growth of a plant may be modified by introducing into a plant an additional copy (in full or in part) of a zinc-finger gene already present in a host plant. The additional gene will silence the endogenous gene, giving rise to a phenomenon known as co-suppression.

Genetic constructs aimed at silencing gene expression may comprise the zinc-finger protein nucleotide sequence, for example as represented by SEQ ID NO 1 (or one or more portions thereof) in a sense and/or antisense orientation relative to the promoter sequence. The sense or antisense copies of at least part of the endogenous gene in the form of direct or inverted repeats may be utilised in the methods according to the invention. The growth of plants may also be modified by introducing into a plant at least part of an antisense version of the nucleotide sequence represented by SEQ ID NO 1. It should be clear that part of the nucleic acid (a portion) could achieve the desired result.

Another method for downregulation of gene expression or gene silencing comprises use of ribozymes, for example as described in Atkins *et al.* 1994 (WO 94/00012), Lenee *et al.* 1995 (WO 95/03404), Lutziger *et al.* 2000 (WO 00/00619), Prinsen *et al.* 1997 (WO 97/3865) and Scott *et al.* 1997 (WO 97/38116).

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Gene silencing may also be achieved by insertion mutagenesis (for example, T-DNA insertion or transposon insertion) or by gene silencing strategies as described by, among others, Angell and Baulcombe 1998 (WO 98/36083), Lowe *et al.* 1989 (WO 98/53083), Lederer *et al.* 1999 (WO 99/15682) or Wang *et al.* 1999 (WO 99/53050). Expression of an endogenous gene may also be reduced if it contains a mutation on the endogenous gene. Such a mutant gene may be isolated and introduced into the same or different plant species in order to obtain plants having modified growth characteristics.

According to a second embodiment of the present invention, genetic constructs and vectors to facilitate introduction and/or expression of the nucleotide sequences useful in the methods according to the invention are provided. Therefore, according to a second embodiment of the present invention, there is provided a construct comprising:

(i) a nucleic acid sequence capable of modulating expression of a nucleic acid encoding a zinc-finger protein and/or activity of a zinc-finger protein;

(ii) one or more control sequence capable of driving expression of the nucleic acid sequence of (i); and optionally

(iii) a transcription termination sequence.

Constructs useful in the methods according to the present invention may be constructed using recombinant DNA technology well known to persons skilled in the art. The gene constructs may be inserted into vectors, which may be commercially available, suitable for transforming into plants and suitable for expression of the gene of interest in the transformed cells. Preferably the genetic construct is a plant expression vector.

The nucleic acid sequence capable of modulating expression of a nucleic acid encoding a zinc-finger protein and/or activity of a zinc-finger protein may be any of the nucleic acid sequences described hereinbefore. A preferred nucleic acid sequence is the sequence represented by SEQ ID NO 1 or a portion thereof or sequences capable of hybridising therewith or a nucleic acid sequence encoding a sequence represented by SEQ ID NO 2 or SEQ ID NO 4 or a homologue, derivative or active fragment thereof.

Plants are transformed with a vector comprising the sequence of interest (i.e., the nucleic acid sequence capable of modulating expression of nucleic acid encoding a zinc-finger protein), which sequence is operably linked to one or more control sequences (at least a promoter). The terms "regulatory element", "control sequence" and "promoter" are all used herein interchangeably and are to be taken in a broad context refer to regulatory nucleic acid sequences capable of effecting expression of the sequences to which they are ligated.

Encompassed by the aforementioned terms are transcriptional regulatory sequences derived from a classical eukaryotic genomic gene (including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence) and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. Also included within the term is a transcriptional regulatory sequence of a classical prokaryotic gene, in which case it may include a –35 box sequence and/or –10 box transcriptional regulatory sequences. The term "regulatory element" also encompasses a synthetic fusion molecule or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ. The terms "control sequence", "regulatory sequence", "regulatory element" and "promoter" are used interchangeably herein. The term "operably linked" as used herein refers to a functional linkage between the promoter sequence and the gene of interest, such that the promoter sequence is able to initiate transcription of the gene of interest.

Advantageously, any type of promoter may be used to drive expression of the nucleic acid sequence depending on the desired outcome. For example, a meristem-specific promoter, such as the rnr (ribonucleotide reductase), cdc2a promoter and the cyc07 promoter, could be used to effect expression in all growing parts of the plant, thereby increasing cell proliferation, which in turn increases yield or biomass. If the desired outcome would be to influence seed characteristics, such as the storage capacity, seed size, seed number, biomass etc., then a seed-specific promoter, such as p2S2, pPROLAMIN, pOLEOSIN could be selected. An aleurone-specific promoter may be selected in order to increase growth at the moment of germination, thereby increasing the transport of sugars to the embryo. A flower-specific promoter, such as pLEAFY, may be utilised if the desired outcome would be to modify the number of flower organs or to produce male-sterile plants. If the desired outcome would be to modify growth and/or developmental characteristics in particular organs, then the choice of the promoter would depend on the organ to be modified. For example, use of a root-specific promoter would lead to increased growth and/or increased biomass or yield of the root and/or phenotypic alteration of the root. This would be particularly important where it is the root itself that is the desired end product, such crops including sugar beet, turnip, carrot, and potato. A fruit-specific promoter may be used to modify, for example, the strength of the outer skin of the fruit or to increase the size of the fruit. A green tissue-specific promoter may be used to increase leaf size. A cell wall-specific promoter may be used to increase the rigidity of the cell wall, thereby increasing pathogen resistance. An anther-specific promoter may be used to produce male-sterile plants. A vascular-specific promoter may be used to increase transport from leaves to seeds. A nodule-specific promoter may be used to increase the nitrogen fixing

capabilities of a plant, thereby increasing the nutrient levels in a plant. A stress-inducible promoter may also be used to drive expression of a nucleic acid to increase membrane integrity during conditions of stress. Particularly preferred stress-inducible promoter include the water stress inducible promoter, WSI18, and the drought stress induced and ABA-related promoter, rab21. Other promoters, inducible under conditions of stress, such as temperature stress (cold, freezing, heat), osmotic stress, drought stress, oxidative stress or biotic stress may also be used in the methods according to the invention.

Preferably, the nucleic acid sequence capable of modulating expression of a gene encoding a zinc-finger protein is operably linked to a constitutive promoter. The term "constitutive" as defined herein refers to a promoter that is expressed continuously. Furthermore, preferably the constitutive promoter is a ubiquitous promoter, which is expressed in more than one, preferably in most or all tissues of the plant. Preferably, the constitutive promoter to be used in the methods of the present invention or to be cloned in the genetic constructs of the present invention is a GOS2 promoter.

Optionally, one or more terminator sequences may also be used in the construct introduced into a plant. The term "terminator" encompasses a control sequence which is a DNA sequence at the end of a transcriptional unit which signals 3' processing and polyadenylation of a primary transcript and termination of transcription. Additional regulatory elements may include transcriptional as well as translational enhancers. Those skilled in the art will be aware of terminator and enhancer sequences which may be suitable for use in performing the invention. Such sequences would be known or may readily be obtained by a person skilled in the art.

The genetic constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type. One example is when a genetic construct is required to be maintained in a bacterial cell as an episomal genetic element (e.g. plasmid or cosmid molecule). Preferred origins of replication include, but are not limited to, the f1-ori and colE1.

The genetic construct may optionally comprise a selectable marker gene. As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention. Suitable markers may be selected from markers that confer antibiotic or herbicide resistance. Cells containing the recombinant DNA will thus be able to survive in the presence of antibiotic or herbicide concentrations that kill untransformed cells. Examples of selectable marker genes include the *bar* gene which

provides resistance to the herbicide Basta; the npt gene which confers resistance to the antibiotic kanamycin; the hpt gene which confers hygromycin resistance. Visual markers, such as the Green Fluorescent Protein (GFP) may also be used as selectable markers. An entire plant may be generated from a single transformed plant cell through cell culturing techniques known to those skilled in the art. Further examples of suitable selectable marker genes include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (nptII), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (gfp) gene (Haseloff *et al.*, Proc. Natl. Acad. Sci. U.S.A, 94 (6), 2122-2127, 1997), and luciferase gene, amongst others.

The present invention also encompasses plants obtainable by the methods according to the present invention. The present invention therefore provides plants obtainable by the method according to the present invention, which plants have modified growth characteristics and which plants have altered zinc-finger protein activity and/or altered expression of a nucleic acid sequence encoding a zinc-finger protein.

According to a third embodiment of the present invention, there is provided a method for the production of transgenic plants having modified growth characteristics, comprising introduction and expression in a plant of a nucleic acid molecule of the invention.

More specifically, the present invention provides a method for the production of transgenic plants having modified growth characteristics, which method comprises:

(i) introducing into a plant or plant cell a nucleic acid sequence or a portion thereof encoding a zinc-finger protein or a homologue, derivative or active fragment thereof;

(ii) cultivating the plant cell under conditions promoting regeneration and mature plant growth.

The protein itself and/or the nucleic acid itself may be introduced directly into a plant cell or into the plant itself (including introduction into a tissue, organ or any other part of the plant). According to a preferred feature of the present invention, the nucleic acid sequence is preferably introduced into a plant by transformation. The nucleic acid sequence is preferably as represented by SEQ ID NO 1 or a portion thereof, or is a nucleic acid sequence encoding an amino acid sequence represented by SEQ ID NO 2 or a homologue, derivative or active fragment thereof. The term "transformation" as referred to herein encompasses the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for transfer. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or

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embryogenesis, may be transformed with a genetic construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem). The polynucleotide may be transiently or stably introduced into a host cell and may be maintained non-integrated, for example, as a plasmid. Alternatively, it may be integrated into the host genome. The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known to persons skilled in the art.

Transformation of a plant species is now a fairly routine technique. Advantageously, any of several transformation methods may be used to introduce the gene of interest into a suitable ancestor cell. Transformation methods include the use of liposomes, electroporation, chemicals that increase free DNA uptake, injection of the DNA directly into the plant, particle gun bombardment, transformation using viruses or pollen and microprojection. Methods may be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. *et al.*, 1882, Nature 296, 72-74; Negrutiu I. *et al.*, June 1987, Plant Mol. Biol. 8, 363-373); electroporation of protoplasts (Shillito R.D. *et al.*, 1985 Bio/Technol 3, 1099-1102); microinjection into plant material (Crossway A. *et al.*, 1986, Mol. Gen Genet 202, 179-185); DNA or RNA-coated particle bombardment (Klein T.M. *et al.*, 1987, Nature 327, 70) infection with (non-integrative) viruses and the like. A preferred method according to the present invention is the protocol according to Hiei *et al.* (Plant J., 6 (2), 271-282, 1994) in the case of rice transformation.

Generally after transformation, plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant-expressible genes co-transferred with the gene of interest, following which the transformed material is regenerated into a whole plant.

Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the gene of interest, copy number and/or genomic organisation. Alternatively or additionally, expression levels of the newly introduced DNA may be undertaken using Northern and/or Western analysis, both techniques being well known to persons having ordinary skill in the art.

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The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformants, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed rootstock grafted to an untransformed scion).

The present invention clearly extends to any plant cell or plant produced by any of the methods described herein, and to all plant parts and propagules thereof. The present invention extends further to encompass the progeny of a primary transformed or transfected cell, tissue, organ or whole plant that has been produced by any of the aforementioned methods, the only requirement being that progeny exhibit the same genotypic and/or phenotypic characteristic(s) as those produced in the parent by the methods according to the invention. The invention also includes host cells containing an isolated nucleic acid molecule encoding a protein capable of modulating a zinc-finger protein, preferably wherein the protein is a zinc-finger protein. Preferred host cells according to the invention are plant cells. The invention also extends to harvestable parts of a plant such as but not limited to seeds, leaves, fruits, flowers, stem cultures, rhizomes, tubers and bulbs.

The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), and plant cells, tissues and organs. The term "plant" also therefore encompasses suspension cultures, embryos, meristematic regions, callus tissue, leaves, seeds, roots, shoots, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily *Viridiplantae*, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia spp., Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica,*

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Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, amaranth, artichoke, asparagus, broccoli, brussel sprout, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugarbeet, sugar cane, sunflower, tomato, squash, and tea, trees and algae amongst others. According to a preferred feature of the present invention, the plant is a monocotyledonous plant, further preferably a cereal, most preferably a plant selected from rice, maize, wheat, barley, soybean, sunflower, canola, sugarcane, alfalfa, millet, barley, rapeseed and cotton.

Advantageously, performance of the methods according to the present invention leads to plants having various economically advantageous modified growth characteristics, such as increased yield/biomass. Accordingly, the present invention relates to methods to alter growth characteristics of a plant or methods to produce plants with altered growth characteristics, wherein the growth characteristics is any one or more of: increased yield, increased biomass, increased total above-ground area, increased plant height, increased number of tillers, increased number of first panicles, increased number of second panicles, increased number of

filled seeds, increased total seed yield per plant, increased harvest index, increased stability of yield modified Tmid, T90 or A42 or plant having an altered growth curve. Increased yield/increased biomass refers to a better performance of a plant under non-stress conditions or under stress conditions compared to the performance of a wild-type plant.

In a preferred embodiment of the present invention, the growth characteristics of a plant are improved when the plant grow under normal or optimal growing conditions, whereby the have sufficient access to all the elements needed to grow and reach maturity. Here the growth characteristics of the plants according to the present ivnetion exceed the growth characteristics of a wild-type plant growing under normal or optimal growing conditions.

Additionally or alternatively, the growth characteristics of a plant may be improved for plant growing under stess conditions. Stress conditions can be any type of environmental, biotic or abiotic stess. Therefore, growth and growth characteristics, biomass production, yield, architecture and survival of plants is also determined by the growing conditions and the way the cell is able to respond (adapt or defend) to such conditions. Factors that influence these agriculturally important characteristics thus include, among others, availability of water, minerals and nutrients, availability of oxygen and CO_2, ozon, temperature, salt concentration, light intensities, presence of competitors or pathogens or symbiont or epiphyte, and occurrence of soil or air pollution. In a particular embodiment of the present invention, the growth characteristics of a plant are improved for plants growing in stress conditions, preferably stress conditions other than cold stress, more preferably for plants growing in salt stress conditions and/or drought stress conditions.

Previously it was described that the SCOF-1 gene of Glycine max is able to enhance cold tolerance to Arabidopsis when overexpressed in that plant. Now it has been surprisingly found that a similar zing-finger gene is able to increase seed yield in a cereal. The methods of the present invention are particularly favorable to be applied to cereals, because the methods of the present invention are used to increase filled seed numbers and /or individual seed weight and/or total seed yield. Also the seed size may be altered by the methods of the present invention. In a particular embodiment of the invention the methods are used to obtain plants with smaller seeds but more seeds. Furthermore, the content of the seeds can altered in the plants of the present invention, for example they can have more starch or more oil, possibly due to their bigger size. Accordingly, a particular embodiment of the present invention relates to a method to increase seed number or seed yield of a cereal, comprising modifying expression of a nucleic acid encoding a zinc-finger protein.

In a particular embodiment of the invention, the methods are used to improve the yield stability of the plants, meaning that year after year, one can obtain the same yield from the progeny of

the plants of the present invention, without too much interference of external factors, such as weather conditions.

The term "modified plant growth" as used herein encompasses, but is not limited to, a faster rate of growth in one or more parts of a plant (including seeds), at one or more stages in the life cycle of a plant, and/or enhanced vigour, and/or aleterd architecture, each relative to corresponding wild-type plants. "Modified architecture" may be due to change in cell division. The term "architecture" as used herein encompasses the appearance or morphology of a plant, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, number, position, texture, arrangement, and pattern of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, leaf, shoot, stem, petiole, trichome, flower, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, sieve element, phloem or vascular tissue, amongst others. Modified architecture therefore includes all aspects of modified growth of the plant. Sometimes plants modify their architecture in response to certain conditions such as stress and pathogens (e.g. nematodes). Therefore, within the scope of the term "architecture" is included modified architecture under conditions such as stress and pathogens.

According to a further preferred feature of the present invention, performance of the methods according to the present invention result in plants having modified architecture. Preferably, the modified architecture is increased plant height, increased number of primary and secondary panicles. Therefore, according to the present invention, there is provided a method for modifying the architecture of plants, which method comprises modulating expression of a nucleic acid sequence encoding a zinc-finger protein and/or modulating activity of a zinc-finger protein in a plant, preferably wherein the zinc-finger protein is encoded by a nucleic acid sequence represented by SEQ ID NO 1,or a portion thereof or wherein the zinc-finger protein is represented by SEQ ID NO 2, or a homologue, derivative or active fragment thereof.

The present invention also relates to use of a nucleic acid sequence encoding a zinc-finger protein and homologues, derivatives and active fragments thereof in modifying the growth characteristics of plants, preferably in increasing yield, further preferably seed yield. The present invention also relates to use of a zinc-finger protein and homologues, derivatives and active fragments thereof in modifying the growth characteristics of plants. The nucleic acid sequence is preferably as represented by SEQ ID NO 1, or a portion thereof or is an amino acid sequence represented by SEQ ID NO 2, or a homologue, derivative or active fragment thereof.

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The present invention also relates to use of a nucleic acid sequence encoding a zinc-finger protein and homologues, derivatives and active fragments thereof and to the zinc-finger protein itself and to homologues, derivatives and active fragments thereof as a growth regulator. The sequences represented by SEQ ID NO 1, and portions thereof and SEQ ID NO 2, and homologues, derivatives and active fragments thereof are useful in modifying the growth characteristics of plants, as hereinbefore described. The sequences would therefore find use as growth regulators, such as herbicides or growth stimulators. The present invention also provides a composition comprising a protein represented by SEQ ID NO 2, or a homologue, derivative or active fragment thereof for the use as a growth regulator.

Conversely, the sequences according to the present invention may also be interesting targets for agrochemical compounds, such as herbicides or growth stimulators. Accordingly, the present invention encompasses use of a nucleic acid sequence as represented by SEQ ID NO 1, or a portion thereof or a sequence represented by SEQ ID NO 2, or homologues, derivatives and active fragments thereof as targets for an agrochemical compound, such as a herbicide or a growth stimulator.

The methods according to the present invention may also be practised by co-expression of a gene encoding a zinc-finger protein in a plant with at least one other gene that cooperates with the gene encoding a zinc-finger protein. Such a gene may be a gene encoding a target protein of the zinc-finger protein. Co-expression may be effected by cloning the genes under the control of a plant expressible promoter in a plant expressible vector and introducing the expression vector(s) into a plant cell using *Agrobacterium*-mediated plant transformation.

The methods according to the present invention result in plants having modified growth characteristics, as described hereinbefore. These advantageous growth characteristics may also be combined with other economically advantageous traits, such as further yield-enhancing traits, tolerance to various stresses, traits modifying various architectural features and/or biochemical and/or physiological features.

The present invention will now be described with reference to the following figures in which:
Fig. 1 is a map of expression vector p3422 for the expression in plants of zinc-finger protein under the control of the GOS2 promoter. CDS1536 is the internal code for the *Arabidopsis* salt tolerant zinc-finger (STZ) protein cDNA. To be expressible in the plant, the zinc-finger protein expression cassette with the GOS2 promoter and a double terminator sequence (T-zein and T-rbcS-deltaGA), is located within the left border (LB repeat) and the right border (RB repeat) of

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the Ti plasmid. Within these T-borders, also a screenable marker and a selectable marker are cloned, each under a constitutive promoter (Prom) and followed by a terminator sequence (polya and t-NOS). Further this vector also contains an origin of replication (pBR322 (ori + bom)) for bacterial replication and a selectable marker (Sm/SpR) for bacterial selection.

Fig. 2A shows digital images from line 81642 positive for the zinc-finger transgene and **Fig. 2B** shows corresponding nullizygotes.

Fig. 3 lists the sequences described in the present application. SEQ ID NO 1 is a STZ encoding nucleic acid sequence wherein the start and the stop codon are highlighted in bold. SEQ ID NO 2 l the protein sequence as encoded by Seq ID NO 1 and the following domains are present in the STZ protein sequence: In bold,italic and underlined a, nuclear localization signal (NLS).In bold and boxed, the 2 zinc finger domains.

Fig. 4A represents a phylogenetic tree of the closest homologues of SEQ ID NO 2 found in plants and **Fig. 4B** represents a phylogenetic tree. The phylogenetic tree was composed using the program Clustal W.

Examples

The present invention will now be described with reference to the following examples, which are by way of illustration alone.

DNA Manipulation

Unless otherwise stated, recombinant DNA techniques are performed according to standard protocols described in (Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York) or in Volumes 1 and 2 of Ausubel *et al.* (1984), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

Example 1: Gene Cloning

The *Arabidopsis* encoding a STZ protein essentially similar to SEQ ID NO 2 was amplified by PCR using as template an *Arabidopsis thaliana* seedling cDNA library (Invitrogen, Paisley, UK). After reverse transcription of RNA extracted from seedlings, the cDNAs were cloned into pCMV Sport 6.0. Average insert size of the bank was 1.5 kb, and original number of clones was of 1.59×10^7 cfu. Original titer was determined to be 9.6×10^5 cfu/ml, after first amplification

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of $6x10^{11}$ cfu/ml. After plasmid extraction, 200 ng of template was used in a 50µl PCR mix. Sequences of the primers used for PCR amplification were, including the attB sites for Gateway recombination (in bold) were PRM3204 (sense, start codon in italics) 5' **GGGGACAAGT TTGTACAAAA AAGCAGGCTT** CACA*ATG*GCG CTCGAGGCTC 3' (SEQ ID NO 3) and PRM3205 (reverse, complementary stop codon in italics) 5' **GGGGACCACTTTGTACAAGAAAGCTGGGT***AAT*TTCCTTAAAGTTGAAGTTTGA 3' (SEQ ID NO 4).

PCR was performed using Hifi Taq DNA polymerase in standard conditions. A PCR fragment of 754 bp was amplified and purifed also using standard methods. The first step of the Gateway procedure, the BP reaction, was then performed, during which the PCR fragment recombines in vivo with the pDONR plasmid to produce, according to the Gateway terminology, an "entry clone", p3359. PDONR was purchased from Invitrogen, as part of the Gateway technology.

Example 2: Vector construction

The entry clone p3359 was subsequently used in an LR reaction with p0640, a destination vector used for rice transformation. This vector contains as functional elements within the T-DNA borders a plant selectable marker and a Gateway cassette intended for LR in vivo recombination with the sequence of interest already cloned in the donor vector. Upstream of this Gateway cassette lies the rice GOS2 promoter for constitutive expression of the zinc-finger gene (De Pater *et al.*, Plant J. 2 (6) 837-844, 1992). After the recombination step, the resulting expression vector p3422 (Figure 1) was transformed into Agrobacterium strain LBA4404 and subsequently to plants.

Example 3: Transformation of a zinc-finger encoding gene into Niponbare

Mature dry seeds of the rice japonica cultivars Nipponbare were dehusked. Sterilization was done by incubating for one minute in 70% ethanol, followed by 30 minutes in 0.2%HgCl2, followed by a 6 times 15 minutes wash with sterile distilled water. The sterile seeds were then germinated on a medium containing 2,4-D (callus induction medium). After incubation in the dark for four weeks, embryogenic, scutellum-derived calli were excised and propagated on the same medium. After two weeks the calli were multiplied or propagated by subculture on the same medium for another 2 weeks. Embryogenic callus pieces were sub-cultured on fresh medium 3 days before co-cultivation (boost of cell division activity). *Agrobacterium* strain LBA4404 harbouring T-DNA vectors comprising a suitable selection marker, were used for cocultivation. *Agrobacterium* was inoculated on AB medium with the appropriate antibiotics and cultured for 3 days at 28°C. The bacteria were then collected and suspended in liquid co-

cultivation medium to a density (OD600) of about 1. The suspension was then transferred to a petri dish and the calli immersed in the suspension for 15 minutes. The callus tissues were then blotted dry on a filter paper and transferred to solidified, co-cultivation medium and incubated for 3 days in the dark at 25°C.

Co-cultivated callus was grown on 2,4-D-containing medium for 4 weeks in the dark at 28°C in the presence of a suitable concentration of the selective agent. During this period, rapidly growing resistant callus islands develop. After transfer of this material to a regeneration medium and incubation in the light, the embryogenic potential was released and shoots developed in the next four to five weeks. Shoots were excised from the callus and incubated for 2 to 3 weeks on an auxin-containing medium from which they were transferred to soil. Hardened shoots were grown under high humidity and short days in a greenhouse. Seeds were then harvested three to five months after transplanting. The method yielded single locus transformants at a rate of over 50 % (Aldemita and Hodges, Planta, 199 612-617, 1996; Chan *et al.*, Plant Mol. Biol. 22 (3) 491-506, 1993, Hiei *et al.*, Plant J., 6 (2) 271-282, 1994).

Example 4: Evaluation of Transformants

Approximately 15 to 20 independent T0 transformants were generated. The primary transformants were transferred from tissue culture chambers to a greenhouse for growing and harvest of T1 seed. Six events of which the T1 progeny segregated 3:1 for presence/absence of the transgene were retained. For each of these events, approximately 10 T1 seedlings containing the transgene (hetero- and homo-zygotes), and approximately 10 T1 seedlings lacking the transgene (nullizygotes), were selected by PCR.

Vegetative growth and seed yield were measured as described above. An increase in the above-ground area, plant height, number of filled seeds, total seed yield per plant, harvest index and number of first panicles, was found in rice plants transformed with the zinc-finger gene compared to control plants (lacking the zinc-finger gene). It is surprisingly found that these beneficial growth characteristics were obtained in plants grown under normal or optimal growing conditions. Whereas another gene of the STZ family, *Glycine max* zinc-finger, was describe to enhance cold-tolerance in transgenic Arabidopsis plants (Kim et al., 2001, Plant J., 25: 247-59) it has now been demonstrated that plants transformed with the zinc-finger gene essentially similar to SEQ ID NO 1, clearly exceeded the performance of the normal growing wild-type plants, and that the effect of the gene was to increase the economical value of normal growing plants.

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(I) Statistical analysis of phenotypic characteristics

A two factor ANOVA (analyses of variance) corrected for the unbalanced design was used as statistical model for the overall evaluation of plant phenotypic characteristics. An F-test was carried out on all the parameters measured of all the plants of all the events transformed with that gene. The F-test was carried out to check for an effect of the gene over all the transformation events and to verify an overall effect of the gene, also named herein "global gene effect". If the value of the F test shows that the data are significant, than it is concluded that there is a "gene" effect, meaning that not only presence or the position of the gene is causing the effect. The threshold for significance for a true global gene effect is set at 5% probability level for the F test.

To check for an effect of the genes within an event, i.e., for a line-specific effect, a t-test was performed within each event using data sets from the transgenic plants and the corresponding null plants. "Null plants" or "Null segregants" or "Nullizygotes" are the plants treated in the same way as the transgenic plant, but from which the transgene has segregated. Null plants can also be described as the homozygous negative transformant plants. The threshold for significance for the t-test is set at 10% probability level. The results for some events can be under or below this threshold. This is based on the hypothesis that a gene might only have an effect in certain positions in the genome, and that the occurrence of this position-dependent effect is not uncommon. This kind of gene effect is also named herein a "line effect of the gene".

The p value is obtained by comparing the t value to the t distribution or alternatively, by comparing the F value to the F distribution. The p value is the probability that the null hypothesis (null hypothesis being "there is no effect of the transgene") is correct. The threshold for significance is set at 5% p-value for the F test and 10% for the t-test.

(II) Vegetative growth measurements

The selected T1 plants (approximately 10 with the transgene and approximately 10 without the transgene) were transferred to a greenhouse. Each plant received a unique barcode label to link unambiguously the phenotyping data to the corresponding plant. The selected T1 plants were grown on soil in 10 cm diameter pots under the following environmental settings: photoperiod= 11.5 h, daylight intensity= 30,000 lux or more, daytime temperature= 28°C or higher, night time temperature= 22°C, relative humidity= 60-70%. Transgenic plants and the corresponding nullizygotes were grown side-by-side at random positions. From the stage of sowing until the stage of maturity (which is the stage were there is no more increase in biomass) the plants were passed weekly through a digital imaging cabinet (examples of

pictures are shown in Figures 2A and 2B). At each time point digital images (2048x1536 pixels, 16 million colours) were taken of each plant from at least 6 different angles. The parameters described below were derived in an automated way from the digital images using image analysis software.

(a) Aboveground area

Results of the maximum above ground area values are summarized in Table 1. The t-test shows that for two of the six events, transgenic plants are significantly larger. The plants of event 84127 were approximately 21% larger. The plants of line 81642 also showed an increase in biomass and an increase of 108 % more above ground biomass, compared to the nullizygotes, was measured for these plants, with a probability value for the populations being equal, of nearly zero.

In all the 6 lines here analyzed, the transgenic plants showed higher or similar area above ground. There was no single line showing a negative effect on this parameter.

When an F-test was carried out on all the plants of all the events it became clear that the transgenic plants show a significant increase in above ground area, in average of approximately 20%, which is statistically highly significant (p value=0.0). The data here presented strongly indicates that the cause for this increase in above ground area for all the transformation events analyzed is due to the introduction in the plants of an STZ gene essentially similar to SEQ ID NO 1.

Table 1: Maximum aboveground area of the events transformed with STZ protein and their segregated null plants.

TotalArea					
Line	TR	null	dif	% dif	p-value
81640	38859	36634	2225	6	0.5598
81642	62092	29903	32190	108	0
84126	48187	47007	1180	3	0.7581
84127	32904	27256	5647	21	0.1405
89257	42978	42840	138	0	0.9711
89268	26936	25982	955	4	0.8139
Overall	42285	35347	6937	20	0

Each row corresponds to one event, for which the average maximum aboveground area (expressed in mm²) was determined for the transgenics and the null lines. P-value stands for the probability produced by the t-test for each event. The last row presents the average numbers for all events. P-value stands for the probability yielded by the F-test. Dif stands for

the difference in absolute values between the two populations for each event, and also globally. % dif is the same parameter expressed in percentage.

(b) Number of primary panicles

The results from the primary panicle counting are summarized in Table 2. The transgenic plants of two events out of six show an increase in primary panicle number (81642 and 84127) compared to their correspondinf nullzygotes, with a probability of the populations being equal of respectively 0.0497 and 0.307.

Table 2: Number of primary panicles for the events transformed with STZ protein.

firstpan					
Line	TR	null	dif	% dif	p-value
81640	3.7	4	-0.3	-8	0.467
81642	4.6	3.8	0.8	22	0.0497
84126	4.7	4.6	0.2	4	0.6784
84127	3.3	2.9	0.5	16	0.307
89257	4.3	4.6	-0.3	-6	0.562
89268	2.9	3	-0.1	-3	0.8382
Overall	3.9	3.8	0.1	4	0.7018

Each row corresponds to one event, for which the number of primary panicles was counted for the transgenics and the null lines. P-value stands for the probability produced by the t-test for each event. The last line presents the average numbers for all events. P-value stands for the probability yielded by the F-test. Dif stands for the difference in absolute values between the two populations for each event, and also globally. % dif is the same parameter expressed in percentage.

(c) Plant height measurements

Results of the plant height measurements are summarized in Table 3. For the transformation event line 81642 it is clearly shown that the presence of a STZ gene in the transgenic plant causes an increase in plant height of at least 16%. Also for line 84127 there is a 9% increase in plant height for the transformed plants, compared to the corresponding nullizygotes.

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Table 3: Maximum plant height observed during the life cycle of transformed rice plants.

Height					
Line	TR	null	dif	% dif	p-value
81640	1033	1002	32	3	0.4529
81642	1136	980	156	16	0.0005
84126	1047	994	53	5	0.2137
84127	990	912	78	9	0.0681
89257	931	971	-40	-4	0.3479
89268	923	907	16	2	0.7264
Overall	1011	963	48	5	0.006

Each row corresponds to one event, for which the average maximum plant height has been determined for the 10 transgenics and the 10 null lines, expressed in mm. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage of difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row presents the average numbers for all four events. There, the p-value stands for the p-value derived from the F-test.

(III) Measurement of seed-related parameters

The mature primary panicles were harvested, bagged, barcode-labelled and then dried for three days in the oven at 37°C. The panicles were then threshed and all the seeds collected. The filled husks were separated from the empty ones using an air-blowing device. After separation, both seed lots were then counted using a commercially available counting machine. The empty husks were discarded. The filled husks were weighed on an analytical balance and the cross-sectional area of the seeds was measured using digital imaging. This procedure resulted in the set of seed-related parameters described below. It was found plants transformed with the STZ gene had more seeds than corresponding control plants. Seed biomass was also increased compared to control plants. Upon analysis, it was found that there was also an increase in the number of filled seeds.

(a) Total number of filled seeds per plant

Total seed number values are summarized in Table 4. The t-test shows that for two of the six events (84127 and 89257), transgenic plants produce 30% and 26% respectively more filled seeds than the nullizygotes. For a third line 81642 which is also identified for increase above ground area and increased plant height and increased number of panicles, it is now demonstrated that there are also increased numbers of filled seeds, with a probability of the

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populations being equal of 0. Therefore it is now demonstrated that the introduction of an STZ gene can be used to increase the number of seeds of a plant, growing in normal growth circumstances, such as a cereal.

Table 4: Total number of filled seeds per plant for the events transformed with STZ protein.

nrfilledseed					
Line	TR	null	dif	% dif	p-value
81640	122.6	162.3	-39.7	-24	0.0514
81642	451	140.5	310.5	221	0
84126	243.3	268.3	-25.06	-9	0.5492
84127	117	90.3	26.75	30	0.1056
89257	147.3	117	30.29	26	0.275
89268	62.5	89.9	-27.4	-30	0.0681
Overall	199.3	152.2	47.08	31	0.0094

Each row corresponds to one event, for which the average total seed number has been determined for the transgenics and the null lines, expressed in units. P-value stands for the probability produced by the t-test for each event. The last line of the table presents the average numbers for all events. P-value stands for the probability yielded by the F-test. Dif stands for the difference in absolute values between the two populations for each event, and also globally. % dif is the same parameter expressed in percentage.

(b) Total seed yield per plant

The total seed yield of the transformed plants are summarized in Table 5. The t-test shows that for two of the events identified for increased number of filled seeds (84127 and 89257), transgenic plants produce significantly more seed weight than the nullizygotes. Also for the third plant line 81642 described for increase number of filled seeds, the transgenic plants show an increased number of filled seeds of 202% compared to the corresponding nullizygote plants of that plant line, with a probability of the populations being equal of nearly 0.

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Table 5: Total seed yield per plant for the events transformed with STZ protein

totalwgseeds					
Line	TR	null	dif	% dif	p-value
81640	3.3	4.4	-1.05	-24	0.0536
81642	10.9	3.6	7.26	202	0
84126	6.5	7	-0.47	-7	0.6159
84127	2.6	2.1	0.51	24	0.175
89257	4	3.3	0.7	22	0.3442
89268	1.5	2.2	-0.68	-31	0.0683
Overall	5	3.9	1.08	27	0.0191

Each row corresponds to one event, for which the average total seed yield has been determined for the transgenics and the null lines, expressed in grams. P-value stands for the probability produced by the t-test for each event. The last line of the table presents the average numbers for all events. P-value stands for the probability yielded by the F-test. Dif stands for the difference in absolute values between the two populations for each event, and also globally. % dif is the same parameter expressed in percentage.

(c) Harvest index

The harvest index in the present invention is defined as the ratio between the total seed yield and the above ground area (mm^2), multiplied by a factor 10^6. Introduction of a nucleic acid essentially similar to SEQ ID NO 1 results in an increase in harvest index. The harvest index values of the STZ-transgenic plants are summarized in table 6. For two lines of which the transgenic plants were identified to have an increase in total seed yield (lines 81642 and 89257), the transgenic plant now show to have also an increase in harvest index of 43% and 18% respectively.

Table 6: Harvest index for the events transformed with STZ protein

totalwgseeds					
Line	TR	null	dif	% dif	p-value
81640	3.3	4.4	-1.05	-24	0.0536
81642	10.9	3.6	7.26	202	0
84126	6.5	7	-0.47	-7	0.6159
84127	2.6	2.1	0.51	24	0.175
89257	4	3.3	0.7	22	0.3442
89268	1.5	2.2	-0.68	-31	0.0683
Overall	5	3.9	1.08	27	0.0191

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Each row corresponds to one event, for which the average harvest index has been determined for the transgenics and the null lines, expressed in grams. P-value stands for the probability produced by the t-test for each event. The last line of the table presents the average numbers for all events. P-value stands for the probability yielded by the F-test. Dif stands for the difference in absolute values between the two populations for each event, and also globally. % dif is the same parameter expressed in percentage

In summary, it can be concluded from the combined results of the plant evaluation, and from the statistical analysis performed on these, that the presence of the STZ transgene essentially similar to SEQ ID NO 1has an effect on above ground area, on plant height, on the number of filled seeds, on the seed yield produced, on the number of first panicles produced, and on the harvest index.

From the evaluation data it is clear that there was a variation between the different transformation events (different plant lines each transformed with the zinc-finger gene). It is well known to persons skilled in the art, for example a plant molecular biologist, that the expression of transgenes in plants, and hence also the phenotypical effect due to expression of such transgene, can differ dramatically among different independently obtained transgenic lines and progeny thereof. The transgenes' present in different independently obtained transgenic plants differ from each other by the chromosomal insertion locus as well as by the number of transgene copies inserted in that locus and the configuration of those transgene copies in that locus. Differences in expression levels can be ascribed to influence from the chromosomal context of the transgene (the so-called position effect) or from silencing mechanisms triggered by certain transgene configurations (e.g. inwards facing tandem insertions of transgenes are prone to silencing at the transcriptional or post-transcriptional level). The exact configuration and insertion loci of the different events have not yet been determined, and expression levels have not been measured. In the some, negative effects may be observed for example in cases when an essential gene is totally silenced instead of being overexpressed (or misexpressed).

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Claims

1. Method for modifying plant growth characteristics, comprising modulating expression in a plant of a nucleic acid sequence encoding a zinc-finger protein and/or modulating activity in a plant of a zinc finger protein.

2. Method for modifying plant growth characteristics, comprising exogenous application of one or more compounds or elements capable of modulating expression of a gene encoding a zinc-finger protein and/or capable of modulating activity of a zinc finger protein.

3. Method for the production of plants, having modified growth characteristics, comprising introducing, into a plant, a nucleic acid sequence capable of modulating activity of a zinc finger protein and/or capable of modulating expression of a zinc-finger gene.

4. Method for producing plants having altered growth characteristics, which method comprises:
   a) Transforming a plant cell with a nucleic acid sequence capable of modulating expression of a gene encoding a zinc-finger protein and/or capable of modulating the activity of a zinc-finger protein;
   b) Cultivating said plant cell under conditions promoting regeneration and mature plant growth.

5. Method according to any of claims 1 to 4, wherein said growth characteristic is any one or more of: increased yield, increased biomass, increased total above-ground area, increased plant height, increased number of tillers, increased number of first panicles, increased number of second panicles, increased number of filled seeds, increased total seed yield per plant, increased harvest index, increased yield stability.

6. Method according to any of claims 1 to 5, wherein the growth characteristics of a plant is improved, said plant growing in growing conditions other than cold-stress conditions, such as under normal or optimal growing conditions, when compared to a wild-type plant growing in the same growing conditions.

7. Method according to any of claims 1 to 6 wherein said zinc-finger protein comprises at least two zinc-finger domains, preferably, wherein said zinc-finger protein is derived from a dicot, further preferably derived from *Arabidopsis*, further preferably wherein

said protein is a STZ protein or a homologue, derivative or functional fragment thereof, further preferably a protein as presented by SEQ ID NO 2.

8. Method according to any of claims 1 to 7 wherein said plant is a monocot plant, preferably a cereal, further preferably a plant selected from rice, maize, wheat, barley, soybean, sunflower, canola, sugarcane, alfalfa, millet, barley, rapeseed and cotton.

9. Method according to any of claims 1 to 8 wherein said gene capable of modulating the activity of zinc-finger protein is driven by a constitutive promoter, such as the GOS2 promoter.

10. Genetic construct comprising:
    a) a nucleic acid sequence capable of modulating expression of a nucleic acid encoding a zinc-finger protein, preferably a protein with at least two zinc-finger domains, further preferably a STZ protein
    b) a control sequence capable of driving expression of the nucleic acid sequence of (a); and optionally
    c) a transcription termination sequence.

11. Genetic construct according to claim 10, wherein said control sequence capable of driving expression of a nucleic acid encoding a zinc-finger protein, is a constitutive promoter, preferably a GOS2 promoter.

12. Host cells containing an isolated nucleic acid molecule encoding a zinc-finger protein, preferably a protein with at least two zinc-finger domains, further preferably a STZ protein, operably linked to a GOS2 promoter.

13. Plant obtainable by a method according to any of claims 1 to 9, which plants have modified growth characteristics.

14. Transgenic plant having altered growth characteristics, containing an isolated nucleic acid molecule encoding a zinc-finger protein.

15. Plant part, preferably harvestable plant part, a propagule or progeny from a plant according to claim 13 or 14.

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16. Use of a nucleotide sequence capable of modulating expression of a nucleic acid encoding a zinc-finger protein or use of a protein capable of modulating the activity of a zinc-finger protein, preferably a protein with at least two zinc-finger domains, further preferably a STZ protein, to alter plant growth characteristics.

17. Use of a nucleotide sequence in breeding programs, which nucleotide sequence is capable of modulating expression of a nucleic acid encoding a zinc-finger protein or capable of modulating the activity of a zinc-finger protein, preferably a protein with at least two zinc-finger domains, further preferably a STZ protein.

18. Use of a zinc-finger gene or a zinc-finger protein, preferably a protein with at least two zinc-finger domains, further preferably a STZ protein as a growth regulator, such as a herbicide or a growth stimulator.

19. Use of a zinc-finger gene or a zinc-finger protein, preferably a protein with at least two zinc-finger domains, further preferably a STZ protein as a target of an agrochemical compound, such as a herbicide or a growth stimulator.

20. Composition comprising a protein essentially similar to SEQ ID NO 2, for use as a growth regulator.

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Abstract

Plants having modified growth characteristics and a method for making the same

The present invention concerns a method for modifying the growth characteristics of plants by modulating expression in a plant of a nucleic acid sequence encoding a zinc-finger protein and/or modulating activity in a plant of a zinc-finger protein. The invention also relates to transgenic plants having modified growth characteristics, which plants have modulated expression of a nucleic acid encoding a zinc-finger protein.

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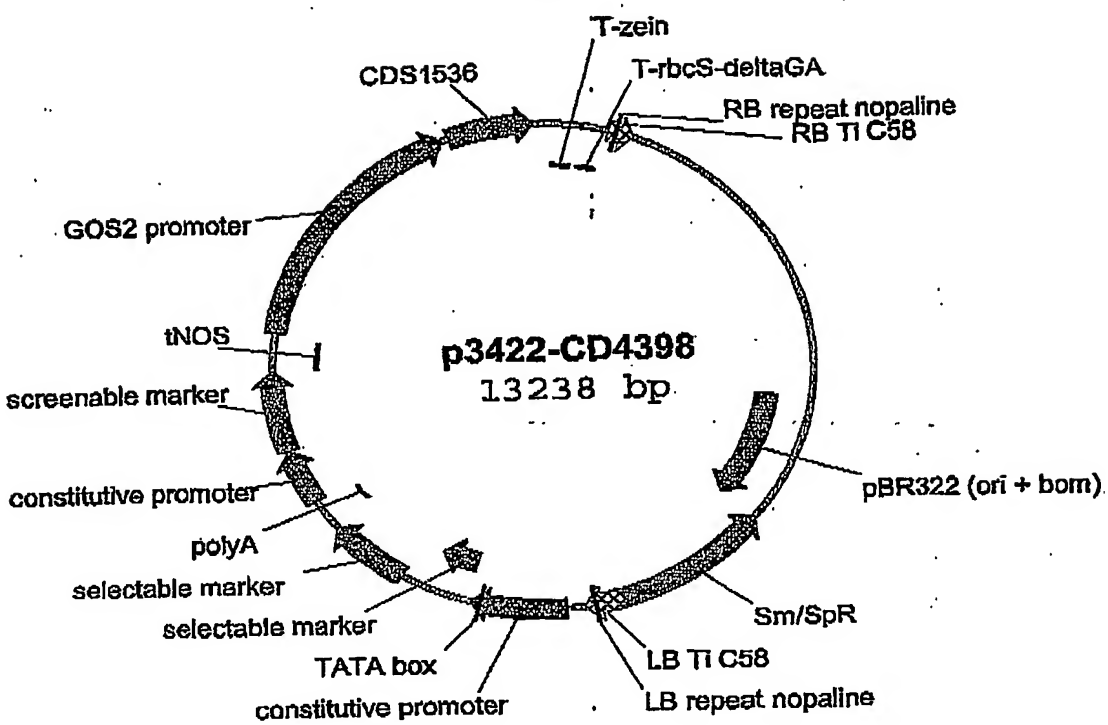

FIGURE 1

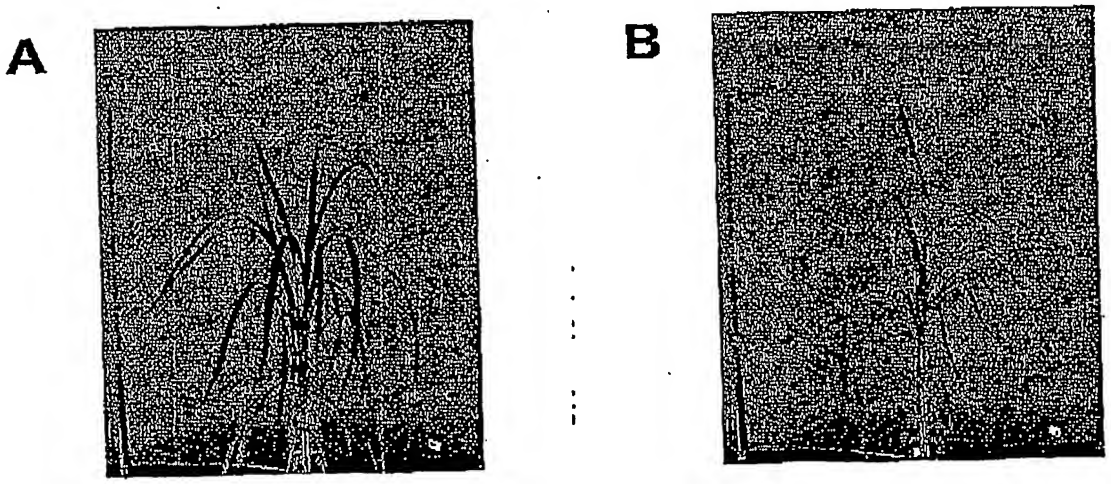

FIGURE 2

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TAAggaaatt

SEQ ID NO 2: Arabidopsis thaliana CDS1536 protein

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SEQ ID NO 2: domains

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SEQ ID NO 3: PRM3204 (sense, start codon in italics)

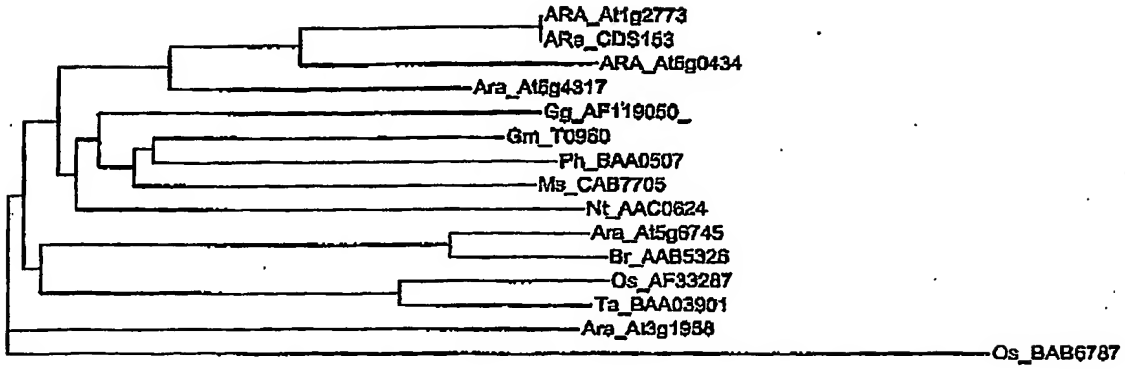
5' GGGGACAAGT TTGTACAAAA AAGCAGGCTT CACAATGGCG CTCGAGGCTC 3'

SEQ ID NO 4: PRM3205 (reverse, complementary stop codon in italics)

5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACAATGGCGCTCGAGGCT 3'

FIGURE 3

A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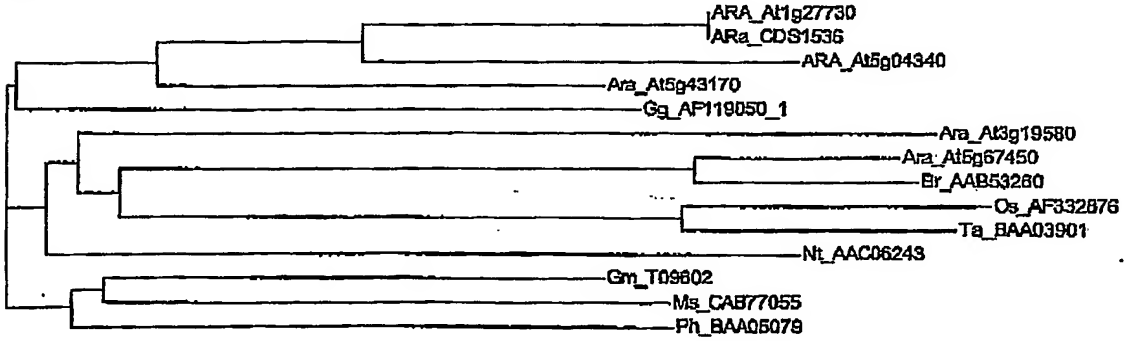
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FIGURE 4

070-STZ-PROV.ST25.txt
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35 40 45

Leu Thr Glu Glu Glu Tyr Leu Ala Phe Cys Leu Met Leu Leu Ala Arg
50 55 60

Asp Asn Arg Gln Pro Pro Pro Pro Pro Ala Val Glu Lys Leu Ser Tyr
65 70 75 80

Lys Cys Ser Val Cys Asp Lys Thr Phe Ser Ser Tyr Gln Ala Leu Gly
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Gly His Lys Ala Ser His Arg Lys Asn Leu Ser Gln Thr Leu Ser Gly
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Thr Thr Gly Ser Gly Lys Ser His Val Cys Thr Ile Cys Asn Lys Ser
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Phe Pro Ser Gly Gln Ala Leu Gly Gly His Lys Arg Cys His Tyr Glu
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